p300/CBP-associated factor (P/CAF) interacts with nuclear respiratory factor-1 to regulate the UDP-*N*-acetyl-α-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-3 gene

Hiroto IZUMI*, Ryo OHTA†, Gunji NAGATANI*, Tomoko ISE*, Yoshifumi NAKAYAMA†, Minoru NOMOTO* and Kimitoshi KOHNO*[1]

*Department of Molecular Biology, School of Medicine, University of Occupational and Environmental Health Japan, Kitakyushu, Fukuoka 807-8555, Japan, and
†Department of Surgery I, School of Medicine, University of Occupational and Environmental Health Japan, Kitakyushu, Fukuoka 807-8555, Japan

We demonstrated recently that expression of the UDP-*N*-acetyl-α-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-3 (GalNAc-T3) gene is restricted to epithelial glands [Nomoto, Izumi, Ise, Kato, Takano, Nagatani, Shibao, Ohta, Imamura, Kuwano, Matsuo, Yamada, Itoh and Kohno (1999) Cancer Res. **59**, 6214–6222]. In the present study, we show that sodium butyrate treatment of human breast cancer MCF-7 cells transcriptionally activates the GalNAc-T3 gene. Transient transfection of plasmids containing a reporter gene under the control of GalNAc-T3 indicated that several transcriptional elements are involved in response to sodium butyrate, with the nuclear respiratory factor-1 (NRF-1)-binding motif located between −88 and −77 nt being the most important. Incubation of a labelled probe encompassing the NRF-1-binding motif with a nuclear extract of sodium butyrate-treated MCF-7 cells yielded a higher level of specific DNA–protein complex versus controls. Flag-tagged NRF-1 expressed in MCF-7 cells can bind to the NRF-1-binding motif of the GalNAc-T3 promoter. Nuclear content of NRF-1 remained constant in MCF-7 cells treated with or without sodium butyrate. Moreover, NRF-1 interacts with and is acetylated by p300/CBP-associated factor (P/CAF). Acetylation of NRF-1 enhances DNA binding. Co-transfection of the GalNAc-T3 reporter plasmid with either NRF-1 or P/CAF expression plasmid resulted in the activation of the GalNAc-T3 promoter. These results indicate a correlation between acetylation of NRF-1 by P/CAF and the butyrate-induced expression of the GalNAc-T3 gene. Additionally, induced expression of P/CAF may be a component of the adenocarcinoma differentiation process.

Key words: acetylation, differentiation, nuclear respiratory factor-1, p300/cAMP-response-element-binding protein, transcription, UDP-*N*-acetyl-α-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-3.

INTRODUCTION

The initial glycosylation of mucin-type O-linked proteins is catalysed by one of the UDP-GalNAc transferases, namely polypeptide *N*-acetylgalactosaminyltransferase [1]. To date, seven distinct human GalNAc transferase genes have been cloned [2–10]. Expression of the UDP-*N*-acetyl-α-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-3 (GalNAc-T3) gene product is highly tissue-specific and is expressed abundantly only in tumour cell lines arising from epithelial glands such as those in breast, colon and prostate tissue [9,11,12].

Extensive analysis of the GalNAc-T3 promoter has shown that GalNAc-T3 gene expression is regulated by multiple systems, including the binding of transcription factors and of a stem and loop structure in the promoter region [11]. This regulation is restricted to cell lines derived from epithelial gland adenocarcinomas [11]. However, we have demonstrated recently that the expression of the GalNAc-T3 is higher in normal colonic mucosa than in colon cancer [13]. This suggests that there may be a correlation between the level of GalNAc-T3 gene expression and the differentiation state of cells derived from epithelial glands. We have also investigated previously whether such an expression influenced tumour differentiation or prognosis in patients with colorectal carcinoma [13]. We found that GalNAc-T3 expression is a novel and useful indicator of tumour differentiation and prognosis [13]. In the present study, we have shown that the differentiating agent, sodium butyrate, can induce expression of the GalNAc-T3 gene. Furthermore, we have shown that nuclear respiratory factor-1 (NRF-1) plays an important role in this induction. NRF-1 was identified originally as a nuclear transcription factor that acts on a number of respiratory proteins [14,15]. It has since been shown that NRF-1 is involved in the transcription of non-respiratory proteins [16–18]. Post-transcriptional modification of the N-terminus of NRF-1 has been shown to play an important role in controlling the DNA-binding activity [19]. Although the precise mechanisms by which sodium butyrate stimulates gene expression through transcription factors remain unclear, co-activators with intrinsic histone acetyltransferase activity may play an important role in butyrate-induced transcription. In the present study, we have demonstrated that p300/CBP-associated factor [P/CAF, where CBP means cAMP-response-element-binding protein (CREB)-binding protein] can interact directly with and modify the N-terminal domain of NRF-1 by acetylation and that the resultant modification stimulates significantly the DNA-binding activity of NRF-1. This finding may have important implications in understanding the molecular mechanism by which acetyltransferase-containing co-activators affect transcriptional activation and cellular differentiation.

Abbreviations used: dn, dominant negative; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; GalNAc-T3, UDP-*N*-acetyl-α-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase-3; GST, glutathione S-transferase; HA, haemagglutinin; HC, human somatic cytochrome *c*; HM, human mitochondrial RNase; NRF-1, nuclear respiratory factor-1; P/CAF, p300/CBP-associated factor; TNT, transcription and translation; wt, wild-type.

[1] To whom correspondence should be addressed (e-mail k-kohno@med.uoeh-u.ac.jp).

EXPERIMENTAL

Materials

Restriction enzymes and other nucleic acid-modifying enzymes were obtained from MBI Fermentas (Vilnius, Lithuania). [α-^{32}P]dCTP, [γ-^{32}P]ATP and [1-^{14}C]acetyl-CoA were from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Synthetic oligonucleotides were obtained from Hokkaido System Science (Hokkaido, Japan). A stock solution (1 mol) of sodium butyrate (Wako, Tokyo, Japan) in distilled water was stored at 4 °C before use.

Cloning of a human NRF-1 and P/CAF cDNA

Full-length cDNAs for human NRF-1 and P/CAF were amplified by reverse transcriptase–PCR using human brain cDNA library (Gibco BRL, Burlington, ON, Canada) and the following primer pairs: NRF-1, 5′-ATGGAGGAACACGGAGTGACCC-3′ and 5′-TCACTGTTCCAATGTCACCACCTCC-3′; P/CAF, 5′-ATGTCCGAGGCTGGCGGGGC-3′ and 5′-TCACTTGTCAATTAATCCAGCTTCC-3′. The PCR products were purified and cloned into pGEM-T Easy (Promega, Madison, WI, U.S.A.).

Preparation of expression plasmids and luciferase reporter plasmids

pT3-Luc4, pT3-Luc5, pT3-Luc6 and glutathione S-transferase (GST)-p53 were prepared as described previously [11]. To obtain Flag-NRF-1wt (where wt stands for wild-type), haemagglutinin (HA)–P/CAF and GST–P/CAF which are expressed in mammalian cells, Flag-tagged NRF-1 and HA-tagged P/CAF cDNA fragments were cloned into the pcDNA3 vector (Invitrogen, San Diego, CA, U.S.A.) and the P/CAF cDNA fragment was cloned into the pGEX4T vector (Amersham Pharmacia Biotech). For Flag-NRF-1dn (where dn stands for dominant negative), which expresses 1–342 amino acids and functions in a dn manner, Flag-NRF-1wt in pcDNA3 vector was digested with *Eco*91I and self-ligated after blunt-end treatment. The pTH-Flag-NRF-1wt plasmid expressing Flag-NRF-1 in *Escherichia coli* was obtained by ligation of a Flag-tagged NRF-1 cDNA fragment into pThioHis vector (Invitrogen) without a ThioHis fragment. HA–p300 and HA–CBP (where CBP stands for CREB-binding protein) derived from cytomegalovirus were gifts from R. Janknecht (Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, U.S.A.). The plasmid DNAs for transient transfection were purified using Qiagen-tip columns (Qiagen, GmbH, Hilden, Germany).

Cell culture and antibodies

Human breast cancer MCF-7 cells were cultured at 37 °C in a humidified atmosphere of 5 % CO_2 in Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan), supplemented with 10 % foetal bovine serum and 0.292 mg/ml L-glutamine. Anti-P/CAF (E-8) and anti-HA (F-7) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-Flag (M2) antibody and anti-HA-peroxidase (3F10) were purchased from Sigma and Roche Molecular Biochemicals (Mannheim, Germany) respectively. Polyclonal antibodies against GalNAc-T3 and NRF-1 were generated by multiple immunizations of a New Zealand white rabbit with synthetic peptides. The sequence of synthetic peptides for GalNac-T3 was as described previously [11], and that for NRF-1 was TMDGQAVEVVTLEQ.

Northern-blot analysis

Northern-blot analysis was performed as described previously [20,21]. Briefly, 20 μg aliquots of total RNA from MCF-7 cells, extracted using Sepasol RNA I (Nacalai tesque, Kyoto, Japan), were subjected to electrophoresis on a 1 % (w/v) agarose gel containing formaldehyde, transferred to a Hybond N$^+$ membrane (Amersham Pharmacia Biotech) and fixed by UV irradiation. The membranes were exposed to ^{32}P-labelled GalNAc-T3 cDNA probes for 24 h at 42 °C and washed twice in 2 × SSC [33.3 mM $C_6H_5Na_3$/33.3 mM NaCl (pH 7.0)], 0.1 % SDS, and twice in 0.2 × SSC/0.1 % SDS, all at 42 °C. Radioactivity was detected with an FLA 2000 image analyser (Fuji Film, Tokyo, Japan).

Transient transfection and luciferase assay

Transient transfection and luciferase assay were performed as described previously [21,22]. Briefly, MCF-7 cells were seeded at a density of 5×10^4 cells/well in a 12-well plate and incubated at 37 °C for 24 h. The cells were then transfected with indicated reporter and expression plasmid DNAs using SuperFect (Qiagen). All cells were co-transfected with 0.3 μg of CH110 expressing β-Gal (Amersham Pharmacia Biotech) and the total amount of plasmid DNA per well was adjusted to 1.2 μg by the addition of mock DNA plasmid. The cells, 3 h after transfection, were rinsed with ice-cold PBS and incubated at 37 °C for 24 h. The cells were then harvested, lysed and centrifuged according to the manufacturer's instructions (Toyoinki, Tokyo, Japan). Luciferase activity in the supernatants was assayed with a Picagene kit (Toyoinki); light intensity was measured for 15 s with a luminometer (Luminescencer-JNRII AB-2300, ATTO, Japan). Results shown were normalized to β-galactosidase activity measured using an enzymic assay kit (Promega) and are representative of at least three independent experiments.

Preparation of nuclear extracts

Nuclear extracts were prepared as described previously [21,22]. Briefly, 2×10^7 MCF-7 cells were treated with 10 mM sodium butyrate for 0–24 h or with 0–20 mM sodium butyrate for 24 h. MCF-7 cells (5×10^4) were transiently transfected with 1 or 3 μg of expression plasmids using SuperFect as described above. Cells were rinsed twice with ice-cold PBS and harvested by scraping into ice-cold PBS. Cells were then pelleted by centrifugation at 500 ***g*** for 5 min and resuspended in ice-cold 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM dithiothreitol (DTT) and 1 mM PMSF. The cells were allowed to swell on ice for 15 min and Nonidet P40 was added to a final concentration of 0.3 %. The homogenate was centrifuged at 2000 ***g*** for 5 min, and the resulting nuclear pellet was re-suspended in ice-cold 20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT and 1 mM PMSF. The samples were rocked gently for 30 min at 4 °C and centrifuged at 21 000 ***g*** for 10 min to remove insoluble material. The resulting supernatant was stored at − 70 °C until use. The protein concentration in each extract was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.), using BSA as the standard.

Binding assay *in vivo* (transient transfection and co-immunoprecipitation assay)

Binding assays *in vivo* were performed as described previously [21,22]. Briefly, MCF-7 cells were seeded in 60 mm plates at a density of 2×10^5 cells/well. The next day, the cells were

co-transfected with 3 μg of HA and Flag-fusion plasmids along with 20 μl of SuperFect according to the manufacturer's instructions (Qiagen). The cells, 3 h after transfection, were washed with PBS, and the medium was renewed. After 48 h, the cells were washed twice with PBS and lysed in buffer X [50 mM Tris/HCl (pH 8.0)/1 mM EDTA/120 mM NaCl/0.5 % Nonidet P40/10 % glycerol and 1 mM PMSF]. After incubating for 30 min on ice, the lysates were centrifuged at 21 000 ***g*** for 10 min at 4 °C. The supernatants (1 mg) were incubated for 60 min at 4 °C with 2 μg of anti-HA (F-7) or anti-Flag (M2) antibody together with 10 μl of Protein A/G–agarose (Qiagen). The beads were then washed three times with binding buffer. The immunoprecipitated samples were separated on 10 % (w/v) SDS gel and developed by Western-blot analysis.

Expression of GST and Flag-fusion proteins in *E. coli*

Expression of recombinant GST-fusion proteins, namely GST–NRF-1, GST–p53 and GST–P/CAF, and pTH-Flag-NRF-1wt was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (Boehringer Mannheim) for 1 h at 25 °C as described previously [21]. The bacteria were collected by centrifugation at 3000 ***g*** for 10 min at 4 °C. The cells were lysed in buffer X and sonicated subsequently for 10 s at 4 °C. After incubating on ice for 30 min, the lysates were centrifuged at 21 000 ***g*** for 15 min at 4 °C and the supernatants stored at − 80 °C until use.

Purification of GST-fusion proteins and translation *in vitro*

To prepare purified GST-fusion proteins, they were immobilized on glutathione–Sepharose beads and eluted with the buffer containing 50 mM Tris/HCl (pH 8.0) and 20 mM GSH according to the manufacturer's instructions (Amersham Pharmacia Biotech). The concentration of GST-fusion proteins was equalized with GST elution buffer. HA–P/CAF, Flag-NRF-1wt and Flag-NRF-1dn were made in a coupled transcription and translation (TNT) system (Promega) using T7 promoter in pcDNA3 vector. Briefly, 0.5 μg of DNA was added directly to 20 μl of TNT rabbit reticulocyte lysate with 0.5 μl of methionine, and reactions were performed at 30 °C for 90 min. The translated products were stored at − 80 °C until use.

Binding assay *in vitro*

Binding assays *in vitro* were performed as described previously [21]. Briefly, GST and GST–P/CAF were immobilized on glutathione–Sepharose beads for 1 h at 4 °C. After the immobilized GST-fusion proteins had been washed three times with Buffer X, soluble Flag-NRF-1wt was added with 1 mM DTT, and further incubated for 4 h at 4 °C. The binding samples were washed three times with Buffer X and developed by Western-blot analysis.

Acetylation *in vitro*

Equal amounts (100 μg) of GST, GST–NRF-1, GST–p53 and GST–P/CAF and 2 μl of Flag-NRF-1wt, Flag-NRF-1dn and HA–P/CAF were made in a coupled TNT system using T7 promoter in pcDNA3 vector. They were then incubated with 1 μl of [1-^{14}C]acetyl-CoA in an acetylation buffer containing 50 mM Tris (pH 8.0), 5 % glycerol, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, 1 mM PMSF and 10 mM sodium butyrate at 30 °C for 1 h [23]. GST-fusion proteins and a half volume of *in vitro* translated proteins were subjected to SDS/PAGE (10 % gel) and immunoblotted with antibodies to Flag or HA respectively. A half volume of *in vitro* translated proteins was then used for electrophoretic mobility-shift assay (EMSA). Radioactivity was detected using an FLA 2000 image analyser (Fuji Film).

Western blotting

Preparation of a whole-cell extract of MCF-7 cells, treated with various concentrations of sodium butyrate, was rinsed twice with ice-cold PBS and harvested by scraping into Buffer X [21]. Cell lysate was centrifuged at 21 000 ***g*** for 10 min at 4 °C and the resulting supernatant was stored at − 70 °C until use. The protein concentration in each extract was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories), using BSA as the standard. Whole-cell lysates, nuclear extracts with or without transient transfection and pull down samples were separated by SDS/PAGE (7.5 or 10 % gel). The proteins were transferred to a PVDF microporous membrane (Millipore, Bedford, MA, U.S.A.) by means of a semi-dry blotter as described previously [20–22]. The blotted membrane was treated with 5 % (w/v) skimmed milk in 10 mM Tris/150 mM NaCl/0.2 % (v/v) Tween 20 and incubated for 1 h at 4 °C with a 1:10 000 dilution of anti-Flag (M2), 1:100 dilution of anti-P/CAF, 1:5000 dilution of anti-GalNAc-T3 and 1:2500 dilution of anti-NRF-1 antibodies. The membrane was then incubated for 30 min at room temperature (25 °C) with the peroxidase-conjugated second antibody or a 1:10 000 dilution of anti-HA-peroxidase. It was then treated with an ECL® kit (Amersham Pharmacia Biotech) and exposed to Kodak X-OMAT film by autoradiography.

EMSA

EMSAs were performed as described previously [21,22]. Briefly, nuclear extract protein from MCF-7 cells or reticulocyte lysates containing Flag-NRF-1wt or Flag-NRF-1dn were incubated for 30 min at room temperature in a buffer containing 10 mM Tris/HCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 8 % glycerol, 1 mM DTT, 0.1 μg poly (dI-dC) and 1×10^4 c.p.m. ^{32}P-labelled oligonucleotide probe. For competition experiments or supershift assays, preincubation for 10 min was performed in the presence of indicated competitor DNA fragment and 0.5 μg of anti-Flag or anti-NRF-1 antibodies. The reaction mixtures were applied to a non-denaturing 4 % (w/v) polyacrylamide gel, which was electrophoresed at 80 V for 2 h in 44.5 mM Tris-borate and 1 mM EDTA. Radioactivity was detected with an FLA 2000 image analyser (Fuji Film). The double-stranded oligonucleotides used for EMSAs were GalNAc-T3 NRF-1 (5′-CTGGCGCGCAGGCGCGGAAG-3′), human somatic cytochrome *c* (HC)–NRF-1 (5′-GCTCGCCAGCATGCGCG-CGC-3′), human mitochondrial RNase (HM)–NRF-1 (5′-CCC-AACGCGCACGCGCACGC-3′), GC-box (5′-TCGATCGGGG-CGGGGCGATCGGGGCGGGGCGA-3′) and nuclear factor κB (5′-TCGAAGGGACTTTCCCAAGGGGACTTTCCCA-3′).

RESULTS

Effect of sodium butyrate on GalNAc-T3 expression

Immunoblotting of cell lysates treated with sodium butyrate revealed that the cellular levels of GalNAc-T3 increased in a time-dependent manner. Enhanced expression of GalNAc-T3 was seen within 12 h and protein levels were 5-fold higher than pretreatment levels after 48 h (Figure 1A). Northern-blot analysis revealed that the amount of GalNAc-T3 mRNA was significantly

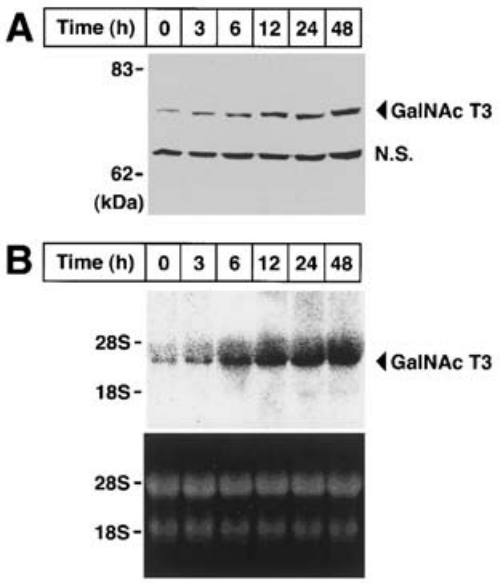

Figure 1 Effect of sodium butyrate on GalNAc-T3 expression

(**A**) MCF-7 cells were treated with 10 mM sodium butyrate for the time period indicated. Whole-cell lysates were subjected to SDS/PAGE (10% gel) and immunoblotted with a polyclonal antibody to GalNAc-T3. The arrowhead denotes the 68 kDa GalNAc-T3 protein. N.S., non-specific band. The prestained molecular-mass marker is shown on the left. (**B**) MCF-7 cells were treated with 10 mM sodium butyrate for the indicated time period. Total RNA samples (20 μg) were electrophoresed on formaldehyde–agarose gels, transferred to Hybond N^+ membranes and hybridized with a 1.5 kb ^{32}P-labelled GalNAc-T3 cDNA fragment. The arrowhead denotes the 4.3 kb GalNAc-T3 mRNA. The lower panel shows loading controls (28 and 18 S RNA bands).

higher 6 h after addition of sodium butyrate, and was increased 4–5-fold relative to control after 48 h (Figure 1B).

Effect of sodium butyrate on GalNAc-T3 promoter activity

We have identified previously specific transcription factor-binding motifs in the GalNAc-T3 gene promoters of several breast cancer cell lines [11]. This suggests that the enhanced GalNAc-T3 mRNA and protein expression in sodium butyrate-treated cells may be due to transcriptional activation. To test whether sodium butyrate can directly activate expression from the GalNAc-T3 promoter, we performed transient transfection assays with plasmids containing various deletions in the promoter (Figure 2). In sodium butyrate-treated cells, the promoter activity of pT3-Luc4, which contains two GC boxes and the NRF-1- and activator protein-2-binding motifs, was approx. 10-fold higher than in untreated cells. Although the promoter activity of pT3-Luc5 in which both GC boxes were deleted was only 30% of that for pT3-Luc4, sodium butyrate also enhanced promoter activity of this plasmid approx. 10-fold. In contrast, sodium butyrate did not alter luciferase expression from the pT3-Luc6 construct in which both GC boxes and the NRF-1-binding motif were deleted. Similar results were obtained when trichostatin A, a specific histone deacetylase inhibitor, was used as a differentiating agent (results not shown).

Effect of sodium butyrate on the NRF-1-binding motif of the GalNAc-T3 promoter

To determine the effect of sodium butyrate on nuclear factors binding to the GalNAc-T3 promoter, EMSAs were performed

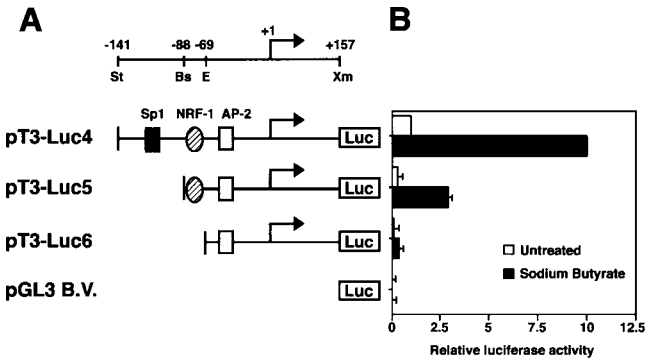

Figure 2 Effect of sodium butyrate on GalNAc-T3 promoter activity in MCF-7 cells

(**A**) Schematic maps of the deletion constructs. The relevant restriction enzyme cleavage sites are: St, *Sty*I; Bs, *Bss*HII; E, *Eco*O1091I; Xm, *Xma*III. Sp1, NRF-1 and activator protein-2 denote the binding motifs of those factors. The arrow indicates the major GalNAc-T3 transcription-initiation site. (**B**) Reporter plasmids (0.9 μg) were transiently transfected into MCF-7 cells. After 24 h, the cells were incubated with or without 10 mM sodium butyrate for a further 24 h. A β-galactosidase reporter gene was co-transfected as an internal control and luciferase activity was normalized to β-galactosidase activity. Each bar represents the average of at least three independent experiments, each performed in triplicate. All results are expressed as a percentage of the corrected luciferase activity of untreated pT3-Luc4.

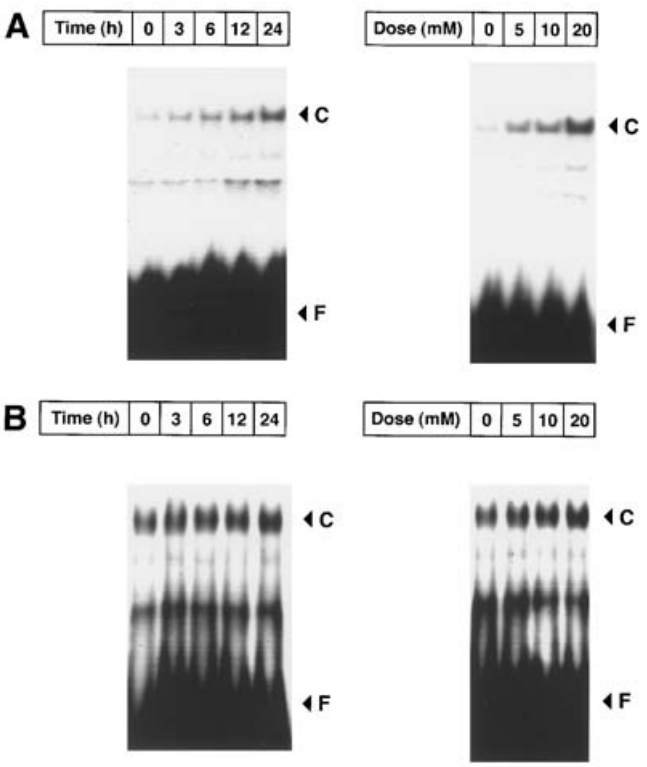

Figure 3 Effects of sodium butyrate on nuclear protein binding to nucleotides as determined using EMSA

Nuclear extracts (10 μg) of MCF-7 cells treated with 10 mM sodium butyrate for 0–24 h or 0–20 mM sodium butyrate for 24 h were incubated with ^{32}P-labelled oligonucleotide including NRF-1-binding motif in the GalNAc-T3 promoter (**A**) and GC-box consensus sequence (**B**). Arrowheads indicate the major DNA–protein complexes (C) and free probe (F).

utilizing a labelled DNA probe containing the NRF-1-binding motif (-88 to -77 nt of the GalNAc-T3 promoter). Whereas a band corresponding to a specific DNA–protein complex was

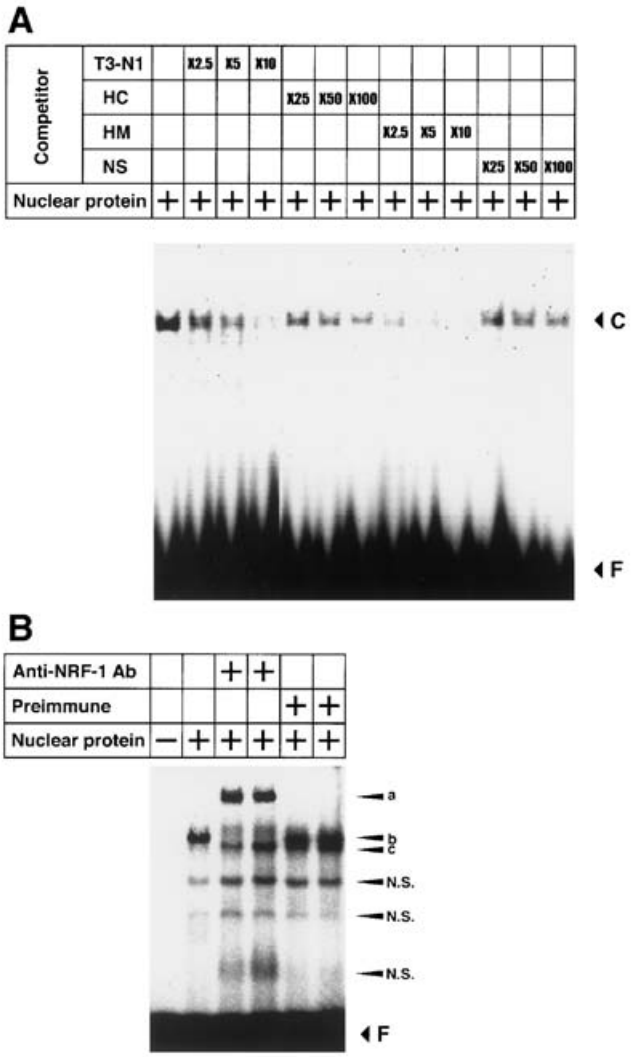

Figure 4 Identification of the binding complex in EMSA

(**A**) Nuclear extracts (10 μg) of MCF-7 cells treated with 10 mM sodium butyrate for 24 h were incubated with the indicated molar excesses of unlabelled oligonucleotides. They were then incubated with a ^{32}P-labelled oligonucleotide including the NRF-1-binding motif in the GalNAc-T3 promoter. HC and HM indicate the NRF-1-binding motif in the HC and HM promoters respectively. Nuclear factor κB consensus sequence was the non-specific competitor (NS). The sequences of the oligonucleotides are as described in the Experimental section. Arrowheads indicate the major DNA–protein complexes (C) and free probe (F). (**B**) Nuclear extracts (10 μg) of MCF-7 cells treated with 10 mM sodium butyrate for 24 h and ^{32}P-labelled oligonucleotide including NRF-1-binding motif in the GalNAc-T3 promoter were incubated with anti-NRF-1 antibody or preimmune IgG. Arrowheads indicate the major DNA–nuclear protein complexes (b), the DNA–nuclear protein–antibody complexes (a), the DNA–serum complexes (c), the DNA–non-specific nuclear protein complexes (N.S.) and free probe (F).

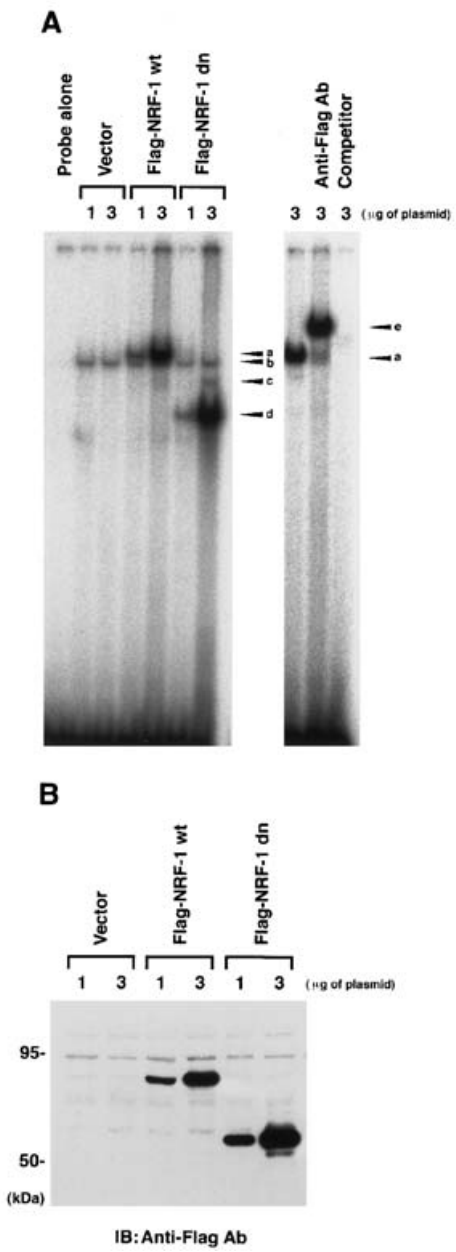

Figure 5 Binding activity of exogenous proteins to NRF-1 motif as determined using EMSA

(**A**) MCF-7 cells (2×10^5) were transiently transfected with 1 or 3 μg of expression plasmids. Nuclear extracts (4 μg) and ^{32}P-labelled oligonucleotide including NRF-1-binding motif in the GalNAc-T3 promoter were incubated with or without anti-Flag antibody. Arrowheads indicate the DNA–Flag-NRF-1wt complexes (a), the DNA–endogenous NRF-1 complexes (b), the DNA–heterodimer of Flag-NRF-1dn and endogenous NRF-1 complexes (c), the DNA–Flag-NRF-1dn complexes (d) and the DNA–Flag-NRF-1wt-antibody complexes (e). (**B**) Nuclear extracts (30 μg) used in this EMSA were subjected to SDS/PAGE (10 % gel) and immunoblotted with an antibody to Flag. The prestained molecular-mass marker is shown on the left.

detected in the nuclear extracts of untreated MCF-7 cells, the level of this complex was significantly higher in extracts from sodium butyrate-treated cells. This increase was dependent on the concentration and time of incubation with sodium butyrate (Figure 3A). Sodium butyrate had little effect, however, when a labelled GC-box oligonucleotide was used as the probe (Figure 3B).

When the nuclear extracts and labelled NRF-1 oligonucleotide were incubated in the presence of homologous unlabelled DNA, the level of specific DNA–protein complex was decreased markedly (Figure 4A). Similar results were observed when an unlabelled oligonucleotide representing the high-affinity NRF-1-binding motif of the HM gene was added. An unlabelled oligonucleotide representing a low-affinity NRF-1-binding motif of the HC gene also disrupted complex formation, but with decreased efficiency (Figure 4A). To investigate whether NRF-1 binds to the NRF-1-binding motif of the GalNAc-T3 promoter, EMSA was performed using a specific antibody to NRF-1. As

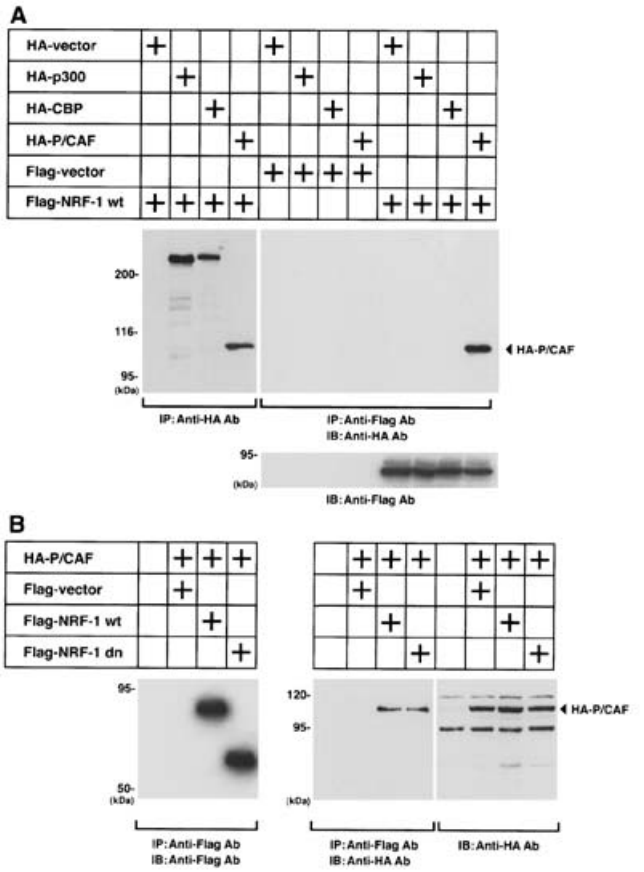

Figure 6 P/CAF interacts with the DNA-binding domain of NRF-1 *in vivo*

(**A**) MCF-7 cells (2×10^5) were transiently transfected with 1.5 μg of Flag or HA expression plasmids. After 48 h transfection, 1 mg of the cell lysate was immunoprecipitated by anti-Flag antibody or 30 μg of the cell lysates was subjected to SDS/PAGE (10 % gel) and immunoblotted with an antibody to Flag or HA. The prestained molecular-mass marker is shown on the left. (**B**) MCF-7 cells (2×10^5) were transiently transfected with 1.5 μg of Flag expression plasmids and HA–P/CAF. Immunoprecipitation and immunoblotting were performed as described above.

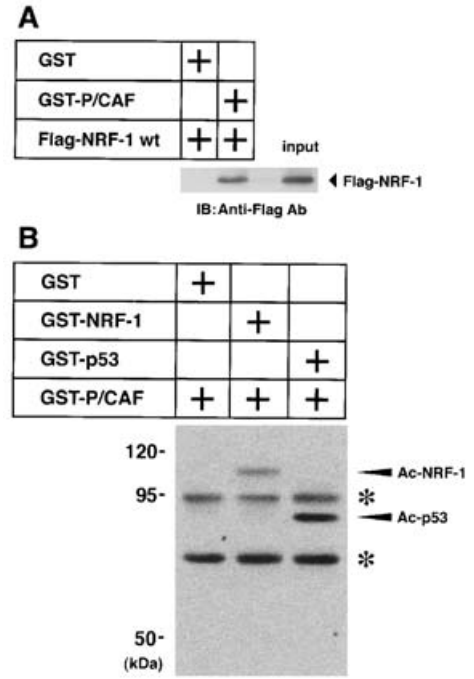

Figure 7 NRF-1 is acetylated by P/CAF by direct interaction

(**A**) Equal amounts of GST alone and GST–P/CAF-fusion proteins, immobilized on glutathione–Sepharose beads, were incubated with HA–p73-fusion protein expressed in bacteria. The bound protein samples and 5 % of the input were subjected to SDS/PAGE (10 % gel) and immunoblotted with an antibody to Flag. (**B**) Purified GST-fusion proteins (100 ng) were incubated with [1-^{14}C]acetyl- CoA and subjected to SDS/PAGE (10 % gel). Arrowheads indicate acetylated GST–NRF-1 (Ac-NRF-1) and acetylated GST–p53 (Ac-p53). * indicates degradation of acetylated GST–P/CAF.

shown in Figure 4(B), the DNA–protein complex was supershifted when an anti-NRF-1 antibody was added, but not when a preimmune antibody was used. Furthermore, nuclear extracts prepared from the cells transfected with expression plasmids for Flag-tagged NRF-1 and -NRF-1dn were used (Figure 5). This allows the expressed protein to be resolved from the endogenous protein and supershifted with a specific monoclonal antibody directed against the tag (Figure 5A). Immunoblotting using antibodies directed against the Flag epitope that is specific for transfected gene products confirmed that Flag-tagged NRF-1 is expressed in a concentration-dependent manner (Figure 5B). Flag-tagged NRF-1 can bind to the NRF-1-binding motif of the GalNAc gene promoter. Three distinct DNA–protein complexes were detected when nuclear extract from the cells transfected with Flag-tagged NRF-1dn was used. The intermediate complex migrating between endogenous NRF-1–DNA complex and Flag-tagged NRF-1dn–DNA complex is derived from the heterodimer–DNA complex (Figure 5A, arrowhead c). The Flag-tagged NRF-1–DNA complex was supershifted by the addition of the antibody, but endogenous NRF-1–DNA complex was not (Figure 5A). Additionally, these DNA–protein complexes were abolished by the addition of an unlabelled oligonucleotide probe.

NRF-1 interacts with P/CAF

A specific DNA–NRF-1 complex was increased significantly when cells were treated with sodium butyrate. However, immunoblotting using antibodies directed against NRF-1 confirmed that the nuclear content of NRF-1 is not increased with sodium butyrate treatment (Figure 9E). DNA-binding activity is commonly enhanced by post-translational modifications such as phosphorylation and acetylation, and sodium butyrate is a potent inhibitor of histone deacetylase. Thus it seemed possible that NRF-1 would be acetylated by interaction with co-activators with intrinsic histone acetyltransferase activity. To determine whether NRF-1 associates with co-activators *in vivo*, we performed co-immunoprecipitation studies on whole-cell extract from MCF-7 cells transfected with various expression constructs. HA–P/CAF was recovered from immunoprecipitates of Flag-NRF-1, but HA–p300 and HA–CBP were not (Figure 6A). HA–P/CAF was also detected in the immunoprecipitated lysate when Flag-NRF-1dn was co-transfected (Figure 6B), suggesting that the N-terminus of NRF-1 may be the interaction domain with P/CAF. NRF-1 also interacts with P/CAF *in vitro* (Figure 7A), suggesting a direct interaction of P/CAF with NRF-1.

P/CAF acetylates NRF-1 and acetylation enhances DNA binding *in vitro*

To examine the possibility that acetylation may modulate DNA-binding activity by NRF-1, we used either purified proteins produced bacterially or translated *in vitro* in the following assays.

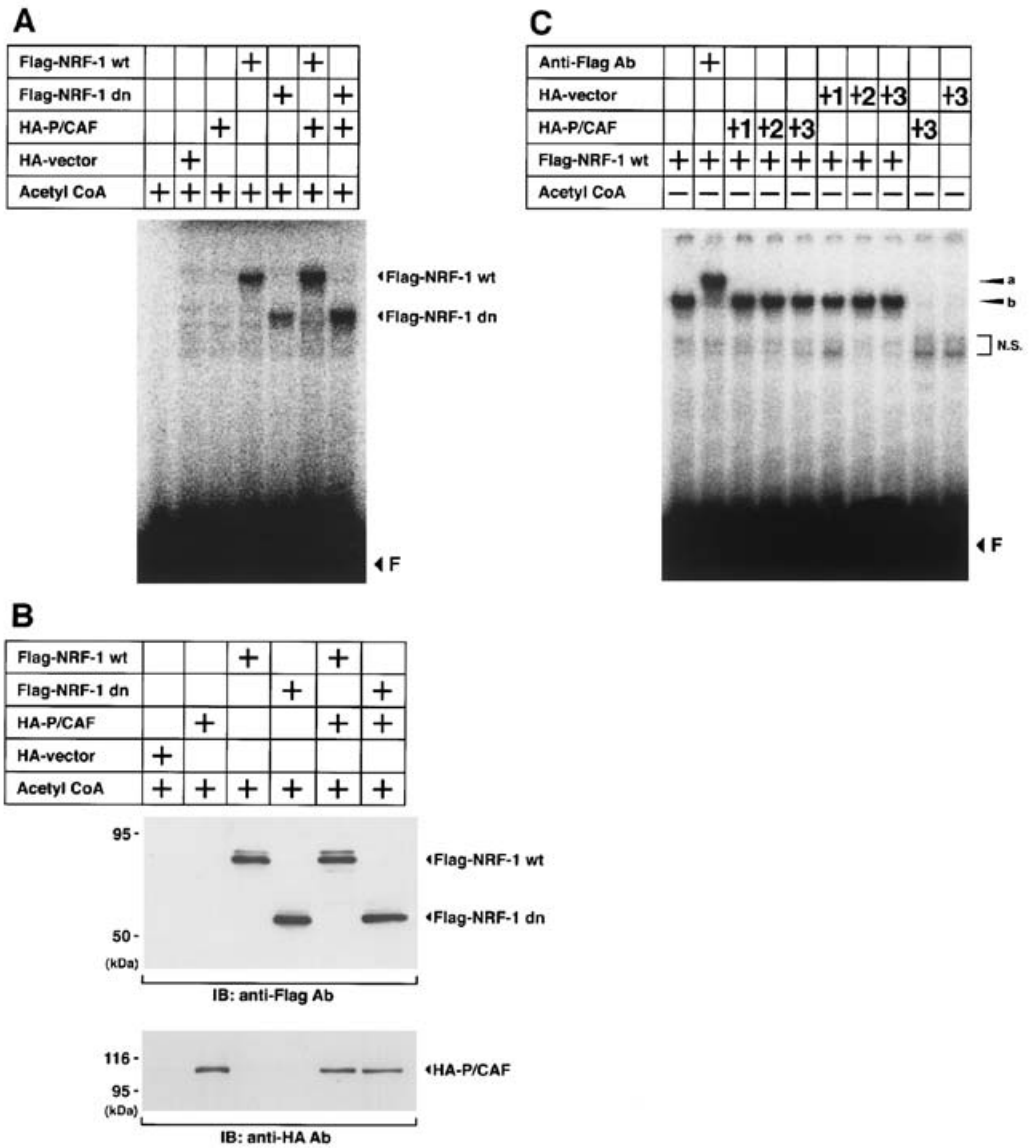

Figure 8 P/CAF acetylates NRF-1 after an increase in the DNA-binding activity

HA–P/CAF, Flag-NRF-1wt and Flag-NRF-1dn were made in a coupled TNT system using T7 promoter in pcDNA3 vector. Acetyl-CoA and 2 μl of translated Flag-NRF-1wt or Flag-NRF-1dn were incubated with or without 2 μl of HA–P/CAF. A sample was used for EMSA with oligonucleotides including the NRF-1-binding motif of the GalNAc-T3 promoter (**A**), and another sample was subjected to SDS/PAGE (10% gel) and immunoblotted with an antibody to Flag or HA (**B**). The prestained molecular-mass marker is shown on the left. Arrowheads indicate free probe (F). (**C**) Flag-NRF-1wt or Flag-NRF-1dn and 2 μl of HA–P/CAF were incubated without acetyl-CoA. The half volume samples were used for EMSA. Arrowheads indicate the DNA–Flag-NRF-1wt complexes (b), the DNA–Flag-NRF-1wt-antibody complexes (a), the DNA–non-specific nuclear protein complexes (N.S.) and free probe (F).

As shown in Figure 7(B), NRF-1 was acetylated by P/CAF as well as p53. However, P/CAF did not increase the DNA-binding activity of a fixed amount of NRF-1 (Figure 8C). Both NRF-1 and NRF-1dn when incubated with P/CAF and acetyl-CoA significantly increased the DNA-binding activity (Figure 8A). The concentrations of NRF-1 and P/CAF used in this assay were equal (Figure 8B), indicating that stimulation of DNA binding was specifically due to acetylation of NRF-1.

NRF-1 and P/CAF transactivate the GalNAc-T3 gene promoter in MCF-7 cells

To determine if the NRF-1 protein or P/CAF directly regulate transcription from the GalNAc-T3 gene promoter, expression plasmids coding for NRF-1 or P/CAF were transfected into MCF-7 cells. Co-transfection of the NRF-1 expression plasmid resulted in an approx. 2.4-fold increase in GalNAc-T3 promoter activity, whereas co-transfection of P/CAF and NRF-1 resulted in an approx. 3.2-fold increase (Figure 9A). Transrepression of the GalNAc-T3 promoter by NRF-1dn was also observed, suggesting that NRF-1dn functions as dn. Figure 9(B) showed the expression levels of three transfected expression plasmids, and are used in Figure 9(A). This result is consistent with a previous report by Solecki et al. [18]. Further studies investigated the ability of P/CAF to potentiate transcription. We found that P/CAF alone transactivated the GalNAc-T3 promoter activity, suggesting that the nuclear concentration or activity of P/CAF is responsible for up-regulating GalNAc-T3 expression by butyrate treatment

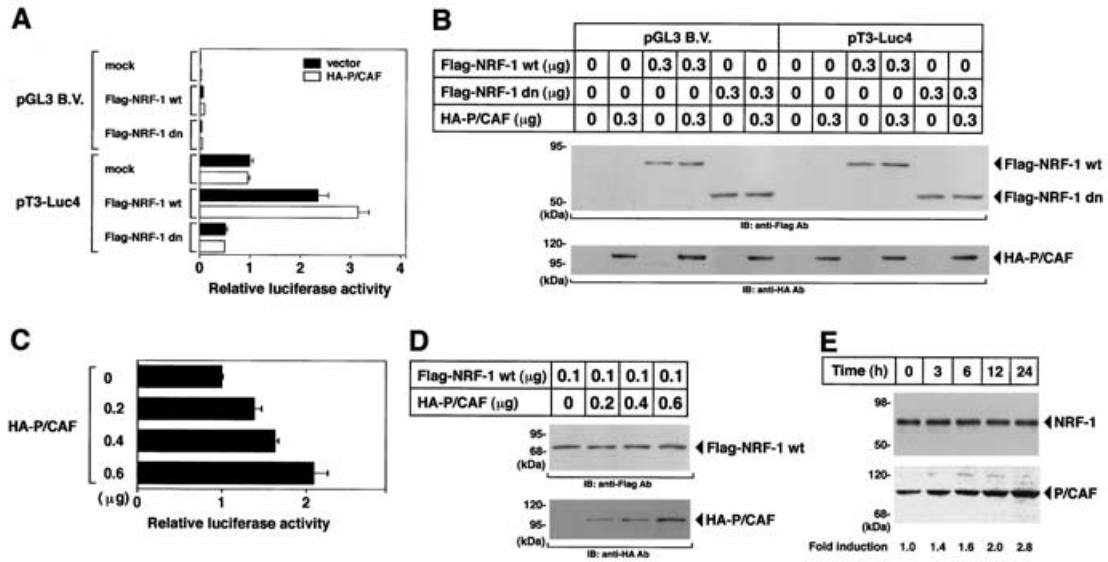

Figure 9 Effect of P/CAF on GalNAc-T3 promoter activity in MCF-7 cells

(**A**) Each sample (0.3 μg) of reporter plasmid and expression plasmids was transiently transfected into MCF-7 cells. After 24 h, the cells were lysed and a luciferase assay was performed. A β-galactosidase reporter gene was co-transfected as an internal control and luciferase activity was normalized to β-galactosidase activity. Each bar represents the average of at least three independent experiments, each performed in triplicate. (**B**) Expression levels of the transfected HA–P/CAF (25 μg of whole-cell extract), Flag-NRF-1wt (25 μg of whole-cell extract) and Flag-NRF-1dn (25 μg of whole-cell extract) determined using luciferase assay (**A**) were shown by immunoblotting with HA or Flag antibody. (**C**) Each sample (0.1 μg) of reporter plasmid, Flag-NRF-1wt plasmid and indicated HA–P/CAF plasmid was transiently transfected into MCF-7 cells. After 24 h, the cells were lysed and luciferase assay was performed as described above. (**D**) Expression levels of the transfected HA–P/CAF (25 μg of whole-cell extract) and Flag-NRF-1wt (50 μg of whole-cell extract) determined using luciferase assay (**C**) were shown by immunoblotting with HA or Flag antibody. (**E**) MCF-7 cells were treated with 10 mM sodium butyrate for the time period indicated. Nuclear extracts (50 μg for NRF-1 and 100 μg for P/CAF) were subjected to SDS/PAGE (10 % gel) and immunoblotted with antibodies to P/CAF and NRF-1. The arrowheads denote the 97 kDa P/CAF and 68 kDa NRF-1 proteins. The prestained molecular-mass marker is shown on the left.

(Figure 9C). Figure 9(D) showed the expression levels of two expression plasmids, and are used in Figure 9(C). Finally, we investigated the effect of sodium butyrate on P/CAF expression. Protein levels of P/CAF were increased significantly when cells were treated with sodium butyrate, whereas levels of NRF-1 remained unchanged (Figure 9E).

DISCUSSION

Mucin plays important roles in both normal physiological processes as well as pathological conditions [24]. The initiation of mucin-type O-linked protein glycosylation is catalysed and regulated by a family of UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases [1]. In comparison with the other GalNAc-T genes, expression of GalNAc-T3 and -T6 has been found to be highly tissue-specific [12,25]. Alteration of mucin-type O-linked glycosylation is also closely related to differentiation phenotypes [3]. Specific O-linked glycosylation and their respective glycosyl transferases may, theoretically, be useful for diagnosis of precancerous and cancerous states.

We have shown previously that GalNAc-T3 is expressed during the early stages of breast cancer, but not in normal mammary gland tissue [11]. Moreover, the level of expression of the GalNAc-T3 gene is high in well-differentiated adenocarcinomas, but low in poorly differentiated adenocarcinomas [12]. GalNAc-T3 gene expression is highly restricted to adenocarcinomas derived from epithelial glands, and several transcription factors have been shown to bind to the promoter region of the GalNAc-T3 gene in breast cancer cell lines [11]. Sodium butyrate, which is present in human plasma and regulates cell growth [26], has been shown to induce the differentiation of various cancer cell lines, including adenocarcinomas *in vitro* [27,28]. We have shown, in MCF-7 cells, that GalNAc-T3 expression was significantly up-regulated by sodium butyrate at both mRNA and protein levels (Figure 1). Transient transfection assays showed that the GalNAc-T3 gene promoter is a butyrate-inducible promoter. We have also shown that both the GC boxes and NRF-1-binding motif and their respective transcription factors are crucial for the basal and butyrate-inducible expression of GalNAc-T3 (Figures 2–5).

NRF-1, in addition to regulating a number of respiratory genes [15,29], may also be an important component in some signal-transduction pathways [30], perhaps regulating growth and development. Footprint analysis *in vivo* showed that the NRF-1-binding motif of the GalNAc-T3 gene was protected completely in MCF-7 cells [11], suggesting that NRF-1 is a constitutive activator required for a basal level of gene expression. EMSA revealed a dramatic increase in the DNA–protein complex formation of NRF-1-binding motif in nuclear extracts from butyrate-treated MCF-7 cells (Figure 3). Furthermore, the GC box appears to be critical for the basal promoter activity. Since sodium butyrate had little effect on the binding of a GC-box oligonucleotide, it is likely that the Sp family is only of minor importance in the regulation of the GalNAc-T3 promoter in differentiated cells. Overexpression of NRF-1 increased the binding activity of the NRF-1-binding motif of GalNAc-T3 (Figure 5). These results indicate clearly that NRF-1 is the transcription factor, which binds to the NRF-1-binding motif of the GalNAc-T3 promoter. Thus butyrate-mediated induction of the GalNAc-T3 gene in MCF-7 cells is manifest via the activity of NRF-1, including basal transcription factors that may require phosphorylation or acetylation. Another possibility is that the unusual structure of the 5′-untranslated region may be involved in the butyrate-induced up-regulation of transcription, since $KMnO_2$-hypersensitive sites have been detected in the 5′-untranslated region of the GalNAc-T3 gene of MCF-7 cells [11].

Sodium butyrate is thought to increase acetylation of transcription factors by inhibiting deacetylase activity. We investigated the possibility of interaction with co-activators which possess intrinsic-acetylase activity and found that P/CAF interacts with NRF-1 through the N-terminal DNA-binding domain (Figures 6 and 7A). Acetylation of NRF-1 and NRF-1dn by P/CAF *in vitro* can enhance the DNA binding (Figures 7B and 8). These results are not unexpected, since 17 lysine residues are clustered in the N-terminal DNA-binding domain. We could not find the amino acid sequences similar to the acetylation sites of p53 and histone. Identification of the acetylated lysine(s) among these residues should be performed with mutant NRF-1 lacking the site or sites of acetylation. In the EMSA, the addition of P/CAF has no effect on the mobility of an NRF-1–DNA complex. Since P/CAF interacts with the DNA-binding site of NRF-1, P/CAF might be dissociated from the complex after acetylation. Serine phosphorylation of the N-terminus of NRF-1 has been shown to enhance DNA binding [19]. It is of interest to examine whether serine phosphorylation is involved in acetylation of NRF-1.

In adenocarcinoma cell lines derived from epithelial tissue, the expression of GalNAc-T3 can be correlated with cellular differentiation [12]. Expression of this gene in colon cancer cell lines can also be induced by sodium butyrate (R. Ohta and K. Kohno, unpublished work), suggesting that GalNAc-T3 gene expression may be a good marker for the biological differentiation of adenocarcinomas. Both NRF-1 and P/CAF can transactivate the GalNAc-T3 gene promoter. We believe this to be the first paper to report that sodium butyrate up-regulates P/CAF expression but not NRF-1 expression (Figure 9E). Up-regulation of P/CAF may stimulate NRF-1 acetylation. To confirm this possibility, further studies including P/CAF knock-down using small interfering RNA and antisense oligonucleotides are required. Our findings suggest that both acetylated NRF-1 and P/CAF expression may be useful in the diagnosis of the differentiation state and/or prognosis of adenocarcinomas.

Sodium butyrate inhibits the growth of human cancer cells, including MCF-7 cells, and triggers apoptosis [31,32]. The NRF-1-binding motif is present in the promoter region of the cytochrome *c* gene [15], which plays an important role in the initiation of apoptosis [33]. Induction of the DNA-binding activity of NRF-1 in butyrate-treated cells may play a role in mediating cell differentiation or cell death by activating target genes.

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